PHARMACOKINETICS AND METABOLISM OF A COX-2 INHIBITOR, VALDECOXIB, IN MICE

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(Received September 30, 2002; accepted January 6, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

The pharmacokinetics and metabolism of valdecoxib, a potent cyclooxygenase-2 selective inhibitor, were investigated in mice. Valdecoxib was extensively metabolized after a single 5 mg/kg oral administration of [14C]valdecoxib and elimination of unchanged drug was minor (less than 1%) in male and female mice. The total mean percentage of administered radioactive dose recovered was 99.8% in the male mice and 94.7% in the female mice. Sixteen metabolites were identified in mouse plasma, red blood cells, urine, and feces. The main phase I metabolic pathway of valdecoxib in mice involved the oxidation of the 5-methyl group to form the active hydroxymethyl metabolite M1. M1 was further oxidized to the carboxylic acid metabolite M4, which underwent opening of the isoxazole ring to form M6 and M13. Phase II metabolism included glucuronide, glucoside, and methyl sulfone conjugations. M1 was also conjugated with glucuronic acid and glucose to yield M-G and M1-glucose, respectively. Three novel methylsulfone conjugates M20, M21, and M21-G were detected in blood or urine. Valdecoxib and M1 were the major radioactive components in plasma and red blood cells. The plasma area under the curve from zero to infinity (AUC0-∞) values for valdecoxib and M1 were 3.58 and 0.850 µg · h/ml in males and 2.08 and 1.63 µg · h/ml in females, respectively. The RBC AUC0-∞ values for valdecoxib and M1 were 12.1 and 22.6 µg · h/g in males and 6.42 and 35.2 µg · h/g in females, respectively.

Valdecoxib, 4-(5-methyl-3-phenylisoxazol-4-yl)benzenesulfonylamine, is a new anti-inflammatory drug that is highly selective for inhibition of the inducible form of cyclooxygenase (COX-2) in vitro enzymatic assays (Talley et al., 2000). This drug (BEXTRA, Pharmacia Corporation) was approved recently by the U.S. Food and Drug Administration for the treatment of rheumatoid arthritis, osteoarthritis, and primary dysmenorrhea (Camu et al., 2002; Fricke et al., 2002). Valdecoxib is the second generation COX-2 inhibitor developed by Pharmacia after celecoxib, the first approved COX-2 inhibitor. These new types of anti-inflammatory drugs are developed based on the hypothesis that selective inhibition of COX-2 should decrease inflammation without the adverse gastrointestinal effects associated with inhibition of the constitutive cyclooxygenase (COX-1) (Donnelly and Hawkley, 1997; Pennisi, 1998; Vane et al., 1998). Clinical studies have demonstrated that COX-2 inhibitors lead to a significant reduction in joint pain, joint tenderness/pain, and joint swelling with a difference in pharmacokinetics of valdecoxib and major metabolites.

Valdecoxib and M1 were the major radioactive components in plasma and red blood cells. The plasma area under the curve from zero to infinity (AUC0-∞) values for valdecoxib and M1 were 3.58 and 0.850 µg · h/ml in males and 2.08 and 1.63 µg · h/ml in females, respectively. The RBC AUC0-∞ values for valdecoxib and M1 were 12.1 and 22.6 µg · h/g in males and 6.42 and 35.2 µg · h/g in females, respectively.

We have recently reported the absorption, distribution, metabolism, and excretion of valdecoxib in humans (Yuan et al., 2002). The primary oxidative metabolic pathways of valdecoxib in humans involved in hydroxylation at either the methyl group to form a hydroxymethyl metabolite or N-hydroxylation at the sulfonamide moiety to form an N-hydroxy metabolite. Further oxidation of the hydroxymethyl metabolite led to the formation of several other phase I metabolites. Oxidative breakdown of the N-hydroxy sulfonamide functional group in the N-hydroxy metabolite led to the formation of corresponding sulfinic acid and sulfonic acid metabolites. The O-glucuronidation of the hydroxymethyl metabolite and N-glucuronidation of valdecoxib were the major metabolites in human urine. The objectives of this study were to determine the total radioactivity recovery in male and female mice following a single oral administration of [14C]valdecoxib at 5 mg/kg, to obtain metabolic profiles in selected mouse plasma RBC, urine, and fecal samples, to identify the major metabolites of valdecoxib, to estimate plasma and RBC pharmacokinetic parameters for total radioactivity, and to examine gender difference in pharmacokinetics of valdecoxib and major metabolites.

Materials and Methods

Chemicals. Valdecoxib and [14C]valdecoxib (uniformly labeled at the six carbons of 3-phenyl ring) were synthesized at Pharmacia Corporation (Skokie, IL). The specific activity of [14C]valdecoxib was approximately 53.6 µCi/mg, and the radiochemical purity was approximately 98.5%, as determined by HPLC with an in-line radioactivity detector. All synthetic standards of the metabolites of valdecoxib were synthesized at Pharmacia Corporation, and their chemical structures were confirmed by both of MS and NMR. All other chemicals and reagents were of analytical grade and available commercially.

Dosing Solution. [14C]Valdecoxib (3.35 mg) and unlabeled valdecoxib (10.2 mg) were dissolved in 11.4 g polyethylene glycol-400 and sonicated for...
approximately 10 min, and then 5.7 g water was added to the mixture. The ratio of polyethylene glycol-400 and water was approximately 2:1, and the final concentration of valdecoxib in the dosing solution was 0.943 mg/g.

Animal Studies. Charles River CD-1 mice (n = 36/sex), purchased from Charles River Breeding Laboratory (Portage, MI), were given [14C]valdecoxib orally at a dose of 5 mg/kg. The radioactive dose for each mouse was approximately 90 μCi/kg. The mice were approximately 8 to 13 weeks of age with body weights of approximately 20 to 40 g. All animals were acclimated and free of any drug treatment prior to the initiation of the study. The mice fasted overnight prior to dosing and for at least 4 h postdose. Following dose administration, the mice were anesthetized with CO2/O2 gas (80:20, v/v). Blood samples (approximately 1 ml) were collected by retro-orbital bleeding (n = 33/sex) into chilled vacutainers containing sodium heparin at 0.5, 1, 2, 4, 6, 24, 48, 72, 96, 120, and 168 h. Three blood samples were collected from each time point, and only one sample was collected from each mouse. Blood samples were kept on ice and centrifuged within 30 min of collection. Plasma was separated from RBC by centrifugation of blood samples at about 3000 g and 4°C for 10 min. All samples were stored at approximately −20°C before analysis.

### TABLE 1

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Measurement</th>
<th>Sex</th>
<th>( T_{max} )</th>
<th>( C_{max} )</th>
<th>AUC(0-( t ))</th>
<th>AUC(0-( \infty ))</th>
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<td>Plasma</td>
<td>Total RAD</td>
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<td>0.5</td>
<td>2.69</td>
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<td>3.52</td>
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<td></td>
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<td>RBC</td>
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</table>

* \( t \) = last time point, for total radioactivity it is 168 h, for valdecoxib and M1 it is 6 h.
* Unit for plasma.
* Unit for RBC.

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FIG. 1. Plasma concentration-time profiles of valdecoxib (SC-65872) and M1 in plasma and RBC following a single oral administration of [14C]valdecoxib (5 mg/kg) to mice.
were dried under a gentle stream of nitrogen at room temperature, and the plasma and RBC samples were 95.0 and 98.5%, respectively. The supernatants were collected and combined. The radioactivity in the combined supernatants was measured by LSC. The LOQ for radioactive dose recovery in urine and feces in mice following a single oral dose of [14C]valdecoxib (5 mg/kg).

<table>
<thead>
<tr>
<th>Male</th>
<th>0-24</th>
<th>0-48</th>
<th>0-72</th>
<th>0-96</th>
<th>0-120</th>
<th>0-144</th>
<th>0-168</th>
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<td>33.3</td>
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<td>36.5</td>
<td>37.0</td>
<td>37.2</td>
<td>37.8</td>
<td>38.0</td>
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<tr>
<td>Feces</td>
<td>52.3</td>
<td>57.6</td>
<td>59.3</td>
<td>60.0</td>
<td>60.8</td>
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<td>97.0</td>
<td>98.1</td>
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<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>43.6</td>
<td>46.0</td>
<td>46.6</td>
<td>46.9</td>
<td>47.1</td>
<td>47.3</td>
<td>47.5</td>
</tr>
<tr>
<td>Feces</td>
<td>42.3</td>
<td>44.6</td>
<td>46.4</td>
<td>46.8</td>
<td>46.9</td>
<td>47.1</td>
<td>47.2</td>
</tr>
<tr>
<td>Total</td>
<td>86.0</td>
<td>90.6</td>
<td>93.1</td>
<td>93.7</td>
<td>94.0</td>
<td>94.5</td>
<td>94.7</td>
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</table>

**TABLE 2** Cumulative percentage of radioactive dose recovery in urine and feces in mice following a single oral dose of [14C]valdecoxib (5 mg/kg).

Analysis. Urine and fecal samples were collected from mice (n = 3/sex) at −18 to 0 h predose and at 24 h intervals for 7 days postdose. Urine was collected by free catch, and feces were collected into preweighed Stomacher bags (A. J. Seward Laboratory, London, England). The samples were stored at approximately −20°C before analysis.

**Total Radioactivity Determination.** **Plasma.** The plasma samples were homogenized by vortexing. A single aliquot (50 µl) was placed in a 20-ml scintillation vial, approximately 10 ml of Aquassure scintillation cocktail (PerkinElmer Life Sciences. Boston, MA) was added, and the radioactivity was determined in a model 1900 TF liquid scintillation counter (LSC) (PerkinElmer Life Sciences). Radioactivity was measured with liquid scintillation spectrometers (Mark III, Tracer Analytic, Elk Grove, IL). Chemical quenching was corrected by the automatic external standard channel ratio method. For all radioactivity measurements, values of radioactivity in DPM less than twice background values were considered below the defined limit of quantitation (LOQ) and reported as zero. Background levels were established after measuring radioactivity in predose samples of the different matrices or samples of acetonitrile. The LOQ value for plasma was 0.02 ng/ml.

**RBC.** The RBC samples were homogenized by vortexing. Duplicate weighed aliquots (approximately 0.1 g) were placed in Centrifuge Cones and pads (PerkinElmer Life Sciences), dried overnight, and oxidized using a model 307 sample oxidizer (PerkinElmer Life Sciences). The combustion products were mixed with 8 ml of Carbosorb and 8 ml of Permaflour (PerkinElmer Life Sciences). The radioactivity in each sample was determined by LSC. The LOQ value for RBC was 0.05 µg.

**Urine.** Total urine volumes were recorded and used in subsequent calculations for percentage of dose excreted in urine. The urine samples were homogenized by vortexing. Duplicate aliquots (100 µl) of urine were placed in 20-ml scintillation vials. Approximately 10 ml of Aquassure liquid scintillation cocktail (PerkinElmer Life Sciences) (1:2, v/v), and carbon-14 radioactivity in the supernatants was measured by LSC. The LOQ value for RBC was 0.05 µg.

**Feces.** Total fecal weights were recorded and used in subsequent calculations for percentage of dose excreted in feces. Fecal samples were mixed with approximately two times the fecal sample weight of a acetonitrile/water mixture (1:1, v/v) in the Stomacher bag and the weights of acetonitrile/water added were recorded. Fecal samples were homogenized using a Stomacher Laboratory Blender 400 (A. J. Seward). Triplicate aliquots (approximately 0.2–0.3 g) of each fecal homogenate were weighed, placed in a Combustone with pads, and combusted using a model 307 sample oxidizer (PerkinElmer Life Sciences). The combustion products were mixed with 8 ml of Carbosorb and 8 ml of Permaflour. The radioactivity in each sample was determined by LSC.

**Quantitative Metabolic Profiling.** **Plasma and RBC.** Due to insufficient volumes, plasma and RBC samples collected at 0.5, 1, 2, 4, 6, and 24 h postdose from three male and female mice were equally pooled resulting in one sample per sex per time point. Each sample was extracted three times with acetonitrile. After each extraction, the samples were centrifuged and the supernatants collected and combined. The radioactivity in the combined supernatant was measured by LSC. The average extraction recoveries for the plasma and RBC samples were 95.0 and 98.5%, respectively. The supernatants were dried under a gentle stream of nitrogen at room temperature, and the residues were reconstituted in mobile phase A and directly injected onto HPLC and LC-MS/MS for analyses. The radioactivity concentration in plasma was calculated based on specific activity of the dosing solution.

**Urine.** Aliquots of urine samples were centrifuged at 2000g for 5 min. The supernatants were injected onto HPLC and LC-MS/MS for analyses. The percentages of the radioactive dose recovered as valdecoxib and metabolites from urine were calculated based on the total radioactivity data combined with HPLC profiling.

**Feces.** The 0–24 h homogenized fecal samples were extracted three times with acetonitrile and two times with mobile phase A to yield an extraction recovery of approximately 75%. The extracts were dried under nitrogen at room temperature and reconstituted in mobile phase A for HPLC profiling. Selected samples were analyzed by LC-MS/MS. The percentages of the radioactive dose recovered as metabolites from feces were calculated based on the total radioactivity data and HPLC profiling.

**β-Glucuronidase Hydrolysis.** Glucuronide conjugates were hydrolyzed by adding approximately 200 µl of β-glucuronidase (Sigma-Aldrich, St. Louis, MO) in 0.5 ml of 0.2 M sodium acetate buffer (pH 5.0; 1:1,v/v). All incubation samples were covered with parafilm and incubated at 37°C for 16 h. The incubation was stopped by addition of 1 ml of ice-cold methanol.

**HPLC.** HPLC analyses of plasma, RBC, urine, and fecal samples were performed using a Hewlett-Packard series 1050 autosampler, a UV detector (λ 240 nm), and a pump (Hewlett Packard Analytical Direct, Wilmington, DE). The separation was carried out on a NovaPak C8 5-µm column (3.9 × 150 mm, 4 µm; Waters, Milford, MA) at ambient temperature with a linear gradient system that was programmed from 100% mobile phase A to 100% mobile phase B in 25 min, followed by isocratic conditions of 100% mobile phase B for 5 min. The system then was programmed back to 100% mobile phase A in 2 min and re-equilibrated for 8 min before the next injection. The flow rate was 1 ml/min. Mobile phase A consisted of acetonitrile/methanol/25 mM ammonium acetate buffer (pH 4.0) (1:2, v/v) and mobile phase B consisted of acetonitrile/methanol/25 mM ammonium acetate buffer (pH 4.0) (1:2, v/v). The eluate from the HPLC column was mixed with Ultima Flo M (PerkinElmer Life Sciences) (1:1, v/v), and carbon-14 radioactivity in the HPLC eluate was detected by a Flo-One[Beta radioactivity detector (Model A-500; PerkinElmer Life Sciences).

**LC-MS/MS.** LC-MS/MS analyses were performed using a system consisting of a PerkinElmer series 200 LC autosampler (PerkinElmer Instruments, Norwalk, CT) and two Shimadzu LC-10A HPLC pumps (Shimadzu, Tokyo, Japan) coupled to a PE Sciex API-III Plus triple quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada). The separation was carried out using the HPLC method as described above. Approximately 20% of a flow rate of 1 ml/min was diverted to the mass spectrometer via a 1:4 T-splitter. The mass spectral analysis was performed in negative electrospray ionization mode. The capillary and orifice voltages were set at −3.7 kV and −65 V, respectively. The nebulizer gas was set at 50 psi. The nitrogen auxiliary and curtain gases were adjusted to a constant flow rate of 2 l/min and 1.8 l/min, respectively. The turbo-ionspray temperature was set at 400°C. Collision induced dissociation (CID) studies were performed using collision energy of 30 eV and at collision gas thickness of 250 × 10^−3 molecules/cm² of argon. LC/Q-TOF analyses were performed using a system consisting of a PerkinElmer series 200 LC autosampler and two Shimadzu LC-10A HPLC pumps coupled to a PE Sciex Qstar quadrupole-time of flight mass spectrometer. The mass spectral analysis was performed in positive electrospray ionization mode set at 400°C with the turbo-ionspray interface and orifice voltages maintained at −3.5 kV and −80 V, respectively. The parent compound valdecoxib was infused in post HPLC column and used as the lock mass. The nitrogen for nebulizer, auxiliary,
and curtain gases were flow controlled at 60 psi, 5 l/min, 1.5 l/min, respectively. CID studies were performed using collision energy of 30 eV.

NMR. The NMR data were acquired using a Bruker DRX-600 600 MHz instrument (Billerica, MA). The sample was dissolved in 180 μl of D$_2$-methanol and placed in a concentric 2.5-mm sample tube. The proton spectrum was determined using a 5-mm indirect detection broadband probe, and the COSY spectrum was determined using a 2.5-mm indirect detection broadband prob.

Pharmacokinetic Analysis. Pharmacokinetic parameters in plasma and RBC for total radioactivity, valdecoxib, and M1 were analyzed by noncompartmental modeling using WinNonlin software (Pharsight, Mountain View, CA). AUC values for plasma and RBC were estimated using the linear trapezoidal method with 1/y weight.

Results

Pharmacokinetics. There were no significant gender differences in the pharmacokinetics of valdecoxib in mice. Mean pharmacokinetic parameters derived from total radioactivity, valdecoxib, and M1 in plasma and RBCs are summarized in Table 1. Plasma and RBC concentration versus time profiles for valdecoxib and M1 are compared in Fig. 1. Valdecoxib and M1 in both plasma and RBC reached the peak levels within 0.5 or 1 h, suggesting that valdecoxib was rapidly absorbed, metabolized, and distributed to blood cells. Concentrations of drug-related radioactivity in plasma and RBC were different in mice. The $C_{max}$ values of total radioactivity, valdecoxib and M1 in RBC were approximately 4, 3, and 15 times higher that those in plasma, respectively. Total exposure (AUCs) of total radioactivity in plasma was similar between female and male mice and approximately 1.4-fold higher in male blood cells as compared with that in female mice. The exposures of valdecoxib in plasma and RBC were generally lower in females, however, these exposures for M1 were found higher in females.

Excretion of Radioactivity after Administration of $[^{14}C]$Valdecoxib. Mean recovery (± S.E.M.) of total radioactivity after oral administration of $[^{14}C]$valdecoxib was 99.8 ± 2.4% in male mice and 94.7 ± 0.6% in female mice (Table 2). In the male mice, 38.0 ± 2.5% of administered dose was excreted in urine and 61.8 ± 3.8% in feces. In the female mice, 47.5 ± 2.2% of administered dose was recovered in urine and 47.2 ± 2.5% in feces.

Metabolic Profiling. Plasma. Representative plasma HPLRC profiles are shown in Fig. 2. Most of the radioactivity in plasma was from valdecoxib and M1. However, minor M11 and M20 were also observed in the mice plasma samples from 6 h postdose. There were no obvious qualitative differences between male and female mice.

RBC. Representative RBC HPLRC profiles are shown in Fig. 3. Most of the radioactivity in RBC was from valdecoxib, M1, and M20,
whereas minor M11 and M21 were also detected. There were no obvious qualitative differences between the RBC profiles of male and female mice.

Urine. Representative urine HPLC profiles for the 0 to 24 h samples are shown in Fig. 4. There were 14 metabolites identified in male and female mouse urine. The major metabolite in male mouse urine was M1-glucose whereas the major metabolites in female mouse urine were M1-glucose, M1-G, and M21-G. Other identified metabolites in mouse urine included M1, M4, M6, M8, M9, M9-G, M10, M10-G, M11, M13, and M15. Only a trace amount of the parent compound (1% of dose) was detected in mouse urine. All metabolites identified in male mouse urine were found in female mouse urine, suggesting there were no qualitative differences between male and female mice.

Feces. Representative fecal HPLC profiles for the 0 to 24 h samples are shown in Fig. 5. The major fecal metabolites in both male and female mice were M1, M10, and M1-G. Valdecoxib was also identified in mouse feces and accounted for less than 1% of administered dose in mice, indicating that the absorption of valdecoxib in mice was complete. Other identified metabolites in mouse feces included M1-glucose, M8, M9, M11, M13, and M15. There were no obvious differences between the male and female fecal profiles.

Identification of Valdecoxib Metabolites. The structures of metabolites were elucidated by electrospray LC-MS/MS using a combination of full and product ion scanning techniques (Prakash et al., 1997; Paulson et al., 2000; Zhang et al., 2000). Some metabolites were further isolated from RBC and urine, then purified and analyzed by NMR. The chemical structures of metabolites were proposed based on MS and NMR data and further confirmed by comparison of their spectra and HPLC retention times to those of synthetic standards when possible. The CID mass spectral data of some metabolites are listed in Table 3.

M1, M9, and M10. The negative-ion electrospray mass spectrometry of M1, M9, and M10 gave the same deprotonated molecular ion [M – H]− at m/z 329, 16 Da higher than valdecoxib, suggesting they were monohydroxylated metabolites of valdecoxib. However, their CID spectra were different. M1 showed a major characteristic fragment ion at m/z 196, which was formed by the loss of C6H5CNOCH2 via a five-member ring rearrangement mechanism on the isoxazole ring. The loss of C6H5CNOCH2 corresponded to the 3-phenyl ring (C6H5), the attached CN group on the isoxazole ring and the 5-methyleneoxide (CH2O), which were confirmed by stable isotopic-labeled compounds (J. Y. Zhang, unpublished data). The fragment ions at m/z 132 and 117 were generated from the losses of 64 (SO2) and 79 Da (H2SO3) from the ion at m/z 196, respectively. Based on MS data, M1 was identified as 4-[5-hydroxymethyl-3-phenylisoxazol-4-yl] benzo-
nesulfonamide. The CID spectrum of M9 yielded fragment ions at m/z 249 and 237 formed by the losses of 79 (SO₂NH) and 92 Da (C₆H₄O) from m/z 329, respectively. The fragment ions at m/z 158 and 157 were generated from the further loss of 80 (SO₂NH₂) or 79 Da (SO₂NH) from m/z 237, respectively. The fragment ion at m/z 118 corresponded to OC₆H₄CN group. This mass spectral fragmentation pattern indicated that the site of hydroxylation occurred on the meta position of the 3-phenyl ring. Therefore, M9 was identified as 4-[3-(3-hydroxyphenyl)-5-methyl-4-isoxazolyl] benzenesulfonamide. In the CID spectrum of M10, the fragment ions at m/z 286, 249 and 237 were formed by the loss of 43 (COCH₃), 80 (SO₂NH₂), and 92 Da (C₆H₄O) from m/z 329, respectively. The further loss of 64 (SO₂), 43 (COCH₃) and 79 Da (SO₂NH) from m/z 286, 249, and 237 produced the fragment ions at 222, 206, and 158, respectively. This mass spectral fragmentation pattern indicated that the site of hydroxylation occurred on the 3-phenyl ring of valdecoxib. M10 was identified as 4-[3-(4-hydroxyphenyl)-5-methyl-4-isoxazolyl] benzenesulfonamide.

The chemical structures of M1, M9, and M10 were further confirmed by comparison of their HPLC retention times and CID mass spectra with the corresponded synthetic standards. M1-G, M9-G, and M10-G. The [M-H]⁻ ions of M1-G, M9-G, and M10-G were different but had some similarity. For examples, they all lost 176 Da (dehydroglucuronic acid) from the deprotonated molecular ion (m/z 505) to generate m/z 329. In addition, they all had the characteristic fragment ions at m/z 113 and 85, which were related to the fragmentation of a glucuronic acid. However, each CID spectrum of M1-G, M9-G, and M10-G showed some very characteristic fragments such as m/z 196 for M1, m/z 237 for M9, and m/z 286, 249, 222 for M10, which were the same as those in their unconjugated metabolites. Based on these results, M1-G, M9-G, and M10-G were identified as O-glucuronide conjugates of M1, M9, and M10, respectively.

M1-glucose. The negative electrospray mass spectrum of M1-glucose showed a [M-H]⁻ ion at m/z 491, 162 Da higher than that of M1, consistent with a glucoside conjugate of M1. The CID spectrum of m/z 491 generated a series of fragment ions at m/z 329, 299, 256, 247, and 196, similar to M1-G. The sequential losses of 162 (dehydroglucose) and 30 Da (CH₂O) from the m/z 491 in the CID spectrum of M1-glucose gave the fragment ions at m/z 329 and 299, respectively, suggesting that the site of glucoside conjugation of M1-glucose occurred at the 5-hydroxymethyl group of M1.

M4, M6, and M8. The mass spectra of M4, M6, and M8 showed [M-H]⁻ ions at m/z 343, 291, and 314, respectively. These metabolites were also detected in human metabolism studies and were confirmed by the comparison with the synthetic standards (Yuan et al., 2002).

M11. The negative-ion mass spectrum of M11 produced a [M-H]⁻ ion at m/z 315, 2 Da higher than that of the parent compound.
valdecoxib. The fragment ions at \( m/z \) 209 and 192 in its CID spectrum were generated from the loss of 42 (CH\(_2\)CO) and 64 (SO\(_2\)) as well as 59 (NH\(_2\)COCH\(_3\)) and 64 Da (SO\(_2\)) from \( m/z \) 315, respectively. The fragment ions at \( m/z \) 58 and 79 corresponded to NHCOCH\(_3\) and SO\(_2\)NH fragments. Based on the MS interpretation, M11 was proposed as an isoxazole ring-open metabolite and was synthesized.

Based on the comparison of the HPLC retention time and the CID spectrum with the synthetic compound, M11 was identified as 4-[(1Z)-1-(aminophenylmethylene)-2-oxopropyl]-benzenesulfonamide. The structure was further confirmed by the synthetic standard.

**M15.** M15 showed a [M – H]\(^{-}\) ion at \( m/z \) 345, 32 Da higher than the parent compound, indicating that a part of the molecule was released during metabolism. In the CID spectrum of M15, the fragment ions at \( m/z \) 286, 265, 253, 235, 222, 207, 195, 180, 156, 134, 118, 100, 78 corresponded to the fragments of SO\(_2\)NH\(_2\), C\(_6\)H\(_5\), and SO\(_2\), respectively. Treatment of M15 with methoxyamine in the presence of pyridine resulted in the disappearance of M15 and a formation of Schiff base derivative with a deprotonated molecular weight of \( m/z \) 303, suggesting the presence of a ketone or aldehyde group. Based on the mass spectral analysis and the chemical derivatization results, M15 was identified as 4-(2-oxo-2-phenylethyl) benzenesulfonamide. The structure was further confirmed by the synthetic standard.

### TABLE 3

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>[M – H]</th>
<th>CID Mass Spectral Data, ( m/z ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>329</td>
<td>196 (100), 132 (52), 117 (98), 78 (68)</td>
</tr>
<tr>
<td>M1-G</td>
<td>505</td>
<td>443 (16), 329 (8), 313 (26), 299 (24), 256 (22), 247 (13), 196 (100), 113 (25), 85 (28)</td>
</tr>
<tr>
<td>M1-glucose</td>
<td>491</td>
<td>329 (8), 313 (4), 299 (34), 256 (25), 247 (27), 196 (100)</td>
</tr>
<tr>
<td>M4</td>
<td>343</td>
<td>196 (82), 132 (47), 117 (100), 78 (48)</td>
</tr>
<tr>
<td>M6</td>
<td>291</td>
<td>184 (100), 120 (62)</td>
</tr>
<tr>
<td>M8</td>
<td>314</td>
<td>271 (10), 234 (14), 222 (10), 207 (35), 192 (47), 183 (13), 173 (15), 145 (12), 119 (100), 80 (68)</td>
</tr>
<tr>
<td>M9</td>
<td>329</td>
<td>249 (15), 257 (80), 158 (77), 157 (100), 118 (29), 75 (56)</td>
</tr>
<tr>
<td>M9-G</td>
<td>505</td>
<td>329 (9), 286 (11), 237 (78), 113 (43), 85 (100)</td>
</tr>
<tr>
<td>M10</td>
<td>329</td>
<td>286 (14), 249 (35), 237 (40), 222 (100), 206 (80), 158 (25), 157 (28), 119 (98), 80 (19)</td>
</tr>
<tr>
<td>M10-G</td>
<td>505</td>
<td>329 (35), 286 (78), 258 (13), 249 (35), 222 (35), 113 (43), 85 (100)</td>
</tr>
<tr>
<td>M11</td>
<td>315</td>
<td>209 (24), 194 (13), 192 (50), 178 (22), 148 (51), 133 (29), 91 (16), 79 (100), 58 (80)</td>
</tr>
<tr>
<td>M13</td>
<td>274</td>
<td>210 (11), 194 (47), 182 (5), 169 (42), 117 (28), 104 (19), 80 (65), 77 (41), 64 (100)</td>
</tr>
<tr>
<td>M15</td>
<td>345</td>
<td>286 (14), 265 (15), 253 (5), 235 (51), 220 (98), 207 (34), 195 (33), 180 (26), 156 (27), 134 (24), 118 (100), 78 (25)</td>
</tr>
</tbody>
</table>
valdecoxib, suggesting the addition of two oxygen atoms. The fragment ions at \( m/z \) 286, 265, and 253 in its CID spectrum were formed by the loss of 59 (CO\(\text{CH}_2\text{OH})\), 80 (SO\(\text{2NH}_2\)), and 92 Da (C\(\text{6H}_2\text{O}\)) from \( m/z \) 345, respectively. The fragment ion at \( m/z \) 235 was generated by a loss of 18 Da (H\(\text{2O}\)) from the \( m/z \) 253. The sequential loss of 45 (CH\(\text{2ONH}\)) or 70 Da (COCON) from the \( m/z \) 265 yielded the fragment ions at \( m/z \) 220 and 195, respectively. The fragment ion of 118 corresponded to O\(\text{C6H4CN}\). Based on the MS data, M15 was tentatively identified as a dihydroxylated metabolite of valdecoxib with hydroxylation at both the para position of the 3-phenyl group and the 5-methyl group on the isoxazole ring.

**M20.** The \([M-H]^-\) ion of M20 was at \( m/z \) 391, 78 Da higher than that of valdecoxib. Q-TOF mass spectrometric analysis showed that M20 had an accurate mass of 391.0420 with elemental composition of C\(\text{17H15N2O5S2}\), which was CH\(\text{2SO2}\) different from valdecoxib, suggesting the addition of a methyl sulfone conjugate. The CID spectrum of M20 yielded fragment ions at \( m/z \) 312, 270, 251, 233, 205, 172, 158, 144, 118, and 79 (Fig. 6). The fragment ions at \( m/z \) 312 and 251 were formed by the loss of 79 (SO\(\text{2NH}\)) or 140 Da (SO\(\text{2C6H4}\)) from \( m/z \) 391. The further losses of 42 (CH\(\text{3CO}\)) or 79 Da (CH\(\text{3SO2}\)) from the \( m/z \) 312 produced the fragment ions at \( m/z \) 270 or 233, respectively. The fragment ion at \( m/z \) 205 was generated from the loss of 46 Da (CH\(\text{3NHO}\)) from \( m/z \) 251. The fragment ion at \( m/z \) 79 corresponded to the SO\(\text{2NH}\) or the SO\(\text{2CH3}\) groups. The fragment ions at \( m/z \) 118, 144, 158, and 172 corresponded to \(\text{NH2SO2C3H2}\), \(\text{NH2SO2C5H4}\), \(\text{NH2SO2C6H6}\), and \(\text{NH2SO2C7H8}\), respectively. The structure of M20 was further confirmed by proton NMR analysis. In the aromatic range, the four protons at C-12, C-13, C-15, and C-16 on the sulfonamide-attached aromatic ring produced the expected A\(\text{2B2}\) pattern, indicating no changes at the aromatic ring (Fig. 8). The patterns of the protons on the 3-phenyl ring support a substitution at the meta position. The protons at C-4 and C-6 were strongly coupled with the proton at C-5 and showed two "doublet" signals. The "triplet" signals were also detected due to the proton at C-5 that coupled with the protons at C-4 and C-6. A "singlet" signal for C-2 was overlapped with the "doublet" signals from the protons at C-3 and C-5. The proton chemical shifts of the 5-methyl group at the isoxazole ring were very similar to the methyl group chemical shift of the valdecoxib standard, indicating no change at the 5-methyl moiety. The methyl group that showed a "singlet" peak at 3.03 ppm was consistent with the chemical shift of the methyl group at the methyl sulfone. The LC-MS/MS and NMR data indicated that M20 was the methyl sulfone.
conjugate of valdecoxib with the site of conjugation occurring at the meta-position on the 3-phenyl ring.

**M21.** Full mass of M21 gave a $[M - H]^{-}$ ion at $m/z$ 407, 16 Da higher than that of M20. The accurate mass of M21 was 407.0372 in its Q-TOF mass spectrum. The elemental composition of C$_{17}$H$_{15}$N$_{2}$O$_{6}$S$_{2}$ was CH$_{2}$SO$_{3}$ different from valdecoxib and one oxygen atom different from M20, suggesting it was a monohydroxylated metabolite of M20. The CID spectrum of M21 showed a series of fragment ions at $m/z$ 270, 196, 132, 117, and 78 (Fig. 7). The fragment ion at $m/z$ 270 was formed by the loss of 137 Da (SO$_{2}$CH$_{3}$/H) from $m/z$ 407. The fragment ions at $m/z$ 196, 132, and 117 were formed by the sequential loss of 147 (C$_{8}$H$_{5}$NO$_{2}$), 64 (SO$_{2}$), and 15 Da (NH) from the deprotonated molecular ion with the same fragmentation mechanism as M1, suggesting the addition of an oxygen atom at the 5-methyl group. The fragment ion at $m/z$ 78 corresponded to the SO$_{2}$CH$_{2}$ group. Based on the MS data, M21 was tentatively identified as the methyl sulfone conjugate of M1, with the site of conjugation occurring at the meta-position on the 3-phenyl ring.

**M21-G.** M21-G showed a $[M - H]^{-}$ ion at $m/z$ 583, 176 Da higher than M21, suggesting that M21-G was a glucuronide conjugate of M21. The CID spectrum of M21-G generated fragment ions at $m/z$ 521, 470, 407, 391, 377, 334, 325, 272, 175, and a base peak at $m/z$ of 196 Da (Fig. 7) that was similar to M1. The sequential loss of 176 (dehydroglucuronic acid), 16 (O), and 14 (CH$_{2}$) Da from the $m/z$ 583 resulted in the fragment ions at $m/z$ 407, 391, and 377, respectively, suggesting that the site of glucuronide conjugation of M21-G occurred at the 5-hydroxymethyl group of M21. The fragment ions at $m/z$ 521 and 470 were formed by the losses of 62 (HOCH$_{2}$CHOH) and 113 (C$_{4}$HO$_{4}$) Da at the glucuronic acid from $m/z$ 583. The fragment ion at $m/z$ 173 corresponded to the dehydroglucuronic acid. Treatment of M21-G with β-glucuronidase resulted in the disappearance of M20-G and the formation of M21, further

![Fig. 7. The CID mass spectra of M21 (top) from mouse RBC and M21-G (bottom) from mouse urine.](image-url)
confirming the glucuronidation. Based on these data, M21-G was tentatively identified as an O-glucuronide conjugate of M21 with the site of conjugation occurring at the 5-hydroxymethyl group.

**Discussion**

After oral administration of 5 mg/kg dose of [14C]valdecoxib to male and female mice, the total recovery of the radioactive dose was near complete, with 99.8% in the males and 94.7% in the females. Approximately equal portions of the radioactivity were recovered in mouse urine (38.1% in males and 47.5% in females) and feces (61.8% in males and 47.2% in females). These results demonstrated that valdecoxib and its metabolites were excreted in mice via both renal and biliary routes.

There was no significant gender difference in the pharmacokinetics of total radioactivity, valdecoxib, and its active metabolite M1 in mice. AUC0–168 h and AUC0–∞ of total 14C were similar, indicating that most of the radioactivity was eliminated from the body within 168 h postdose. RBC pharmacokinetic values were considerably greater than plasma values, indicating that valdecoxib, M1, and other metabolites may be preferentially partitioned into RBC.

Both male and female mice extensively metabolized valdecoxib after oral administration, with less than 1% of the radioactive dose excreted as the unchanged drug by either sex. A total of 16 metabolites were identified in mice with nine of them unreported before. The plausible scheme for the biotransformation pathways of valdecoxib in mice is shown in Fig. 8.

One of the major routes of the metabolism involved oxidation at the 5-methyl group on the isoxazole ring to form a 5-hydroxymethyl metabolite M1. The conjugation of M1 with glucuronic acid and glucose yielded the O-glucuronide conjugate (M1-G) and the O-glucoside conjugate (M1-glucose). In mice M1 was the major phase I metabolite. In urine M1 was mainly excreted as M1-G and M1-glucose, accounting for 4 and 14% of the dose in male mice and 13 and 12% of the dose in female mice, respectively. In feces M1 was mainly excreted as M1 and M1-G, accounting for 22 and 5% of the dose in male mice and 8 and 5% of the dose in female mice.
respectively. In combination of urine and feces M1 and its conjugates accounted for approximately 45 and 38% of the dose in male and female mice, respectively. M1 and the parent drug valdecoxib were the major radioactive components circulating in blood. The further oxidation of the 5-hydroxymethyl group of M1 resulted in the formation of the carboxylic acid metabolite M4, which underwent the isoxazole ring-opening and the subsequent loss of two carbon dioxide by oxidation followed by the reduction of the double bond to form metabolite M6. M13 was assumed to be the deamination and dehydration product of M6 as proposed in Fig. 8.

Another major route of metabolism was oxidation of the 3-phenyl ring to form an epoxide intermediate followed by the hydrolysis to form the meta- and the para-hydroxyphenyl metabolites M9 and M10, or followed by glutathione adduction to yield the meta-gluthathione S-conjugate P-SG. The glucuronidation of M9 and M10 gave the O-glucuronide conjugates M9-G and M10-G, respectively. M1 or M10 underwent additional oxidation to form the dihydroxylated metabolite M15. P-SG was not detected in mice but in rat bile along with M10 underwent additional oxidation to form the dihydroxylated metabolite M15. P-SG was not detected in mice but in rat bile along with its 5-hydroxylated metabolite (M1-G) after administration of valdecoxib (J. Y. Zhang, unpublished works). However, three methylsulfone S-conjugates (M20, M21, and M21-G) were detected in mouse blood or urine. The mechanism for the formation of these novel methylsulfone conjugates is not known at this time and needs to be further investigated. One of the explanations may involve the breakdown of the glutathione S-conjugate P-SG by γ-glutamyltransferase and dipeptidase to form the cysteine S-conjugate, which is then cleaved by renal cysteine conjugate β-lyase to give the thio-conjugate. The methylation of the thio-conjugate yields the thiomethyl derivative and is then further oxidized to form the methylsulfone S-conjugate M20. The additional oxidation of M20 at the 5-methyl group results in the formation of M21, which undergoes the subsequent glucuronidation to form the O-glucuronide conjugate M21-G. The enzymatic processing for degradation of glutathione S-conjugate to yield thio-conjugate is well understood (Dekant et al., 1994). The evidence to support this mechanism derived from the detection of the cysteine and mercapturate S-conjugates of valdecoxib in rabbit urine (J. Zhang, unpublished works). Moreover, a similar pathway for formation of a methylsulfone metabolite from a thio compound has been shown in previous reports (Karim and Brown, 1972; Karim et al., 1975). M20 and M21 were found in mouse plasma and RBC only, whereas M21-G was observed in mouse urine. In the later time points of RBC samples, M20 was the only metabolite detected. It seems likely that the hydrophobic compound M20 has to be further oxidized to form a polar compound M21 and is then excreted as a glucuronide conjugate (M21-G) in urine. M21-G accounted for approximately 5 and 16% of administered radioactive dose in male and female mice, respectively.

The other minor metabolites were caused by N-hydroxylation of the sulfonamide (M8) and reduction on the isoxazole ring (M11). N-hydroxylation of valdecoxib at the sulfonamide moiety formed the putative N-hydroxy sulfonamide metabolite (M2) that was unstable metabolically and not found in mice. However, the oxidative breakdown of the N-hydroxy sulfonamide group in M2 led to the formation of a sulfonic acid metabolite M8, which was detected in mouse urine. Both M2 and M8 were found in human urine (Yuan et al., 2002). Reduction of valdecoxib on the isoxazole ring resulted in the formation of an isoxazole ring-opened metabolite M11.

In conclusion, near-completed recovery of radioactivity was observed after oral administration of [14C]valdecoxib in mice. Sixteen metabolites were identified in mice with nine novel metabolites including three methylsulfone conjugates. M1, its phase II metabolites (M1-G and M1-glucose) and M21-G were the major excretion products. Valdecoxib was well absorbed and extensively metabolized with minimal parent compound detected in mouse urine and feces. There were no significant gender differences for the pharmacokinetics and metabolism of valdecoxib in mice.

Acknowledgments. We thank Fran Xu and Caroline Dudkowski for skillful technical assistance.

References